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Attn: TSCA 8(e) Coordinator

RE: TSCA 8(e) Document Control Number 8EHQ-0497-13581

TSCA 8(e) Substantial Risk
Bisphenol-A
CAS No. 80-05-7

At the request of General Electric Company, the enclosed report titled *Evaluation of Reproductive Organ Development in CF-1 Mice Following Prenatal Exposure to Bisphenol A* is supplemental to General Electric Company's previous TSCA 8(e) submission on Bisphenol-A, TSCA 8(e) Document Control Number 8EHQ-0497-13581, dated January 29, 1996. Also enclosed is a copy of a cover letter we have sent to Dr. Frederick vom Saal at the University of Missouri, Columbia in light of his interest and assistance with this matter. You will note that we have sent him the text portion of the report.

Please do not hesitate to contact me if you have any questions at (202) 974-5217.

Sincerely,

Lynne R. Harris

Lynne R. Harris
Staff Director
Bisphenol A Global Industry Group

8EHQ-96-13581

Enc. (2)

89990000022

cc: Dr. Lynne Goldman, EPA
BPA Technical Steering Committee
Stephen F. Austin, General Electric Company

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October 14, 1998

Frederick vom Saal, Ph.D.
Division of Biological Sciences
University of Missouri-Columbia
105 Lefevre Hall
Columbia, MO 65211

Dear Fred,

We finally received the signed report from the contract laboratory on SPI's research involving bisphenol A and mice.

I am enclosing a copy of the text portion of the final report and will forward a manuscript to you shortly. We appreciate the assistance you and others associated with the University of Missouri were to us in developing the research protocol.

Best Regards,

Lynne R. Harris/gBH

Lynne R. Harris
Staff Director
Bisphenol A Global Industry Group

Enc.

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of the Plastics

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Teamwork to achieve the highest standard in research

EVALUATION OF REPRODUCTIVE ORGAN DEVELOPMENT IN CF-1 MICE FOLLOWING PRENATAL EXPOSURE TO BISPHENOL A

TEST ARTICLE:	4,4'-isopropylidene-2-diphenol (Bisphenol A, BPA)
PERFORMING LABORATORY:	MPI Research 54943 North Main Street Mattawan, MI 49071-9399 USA (616) 668-3336
LABORATORY STUDY IDENTIFICATION:	328-046
STUDY DIRECTOR:	William J. Breslin, Ph.D.
SPONSOR ADDRESS:	The Society of the Plastics Industry, Inc. Bisphenol A Task Group Suite 600 1801 K Street, N.W. Washington, D.C. 20005-1301
SPONSOR REPRESENTATIVE:	Mr. Lynne R. Harris
DATE OF STUDY COMPLETION:	October 9, 1998

STATEMENT OF COMPLIANCE

This non-clinical laboratory study was conducted in accordance with the United States Environmental Protection Agency, Toxic Substance Control Act Good Laboratory Practice Standards, 40 CFR Part 792, the United States Food and Drug Administration Good Laboratory Practice Regulations, 21 CFR Part 58, Final Rule effective October 5, 1987, the Japanese Ministry of International Trade and Industry (MITI), GLP Standards Applied to Industrial Chemicals, and the Organization for Economic Cooperation and Development (OECD), the OECD Principles of Good Laboratory Practices, as specified by the European Economic Community, Council Directive 87/18 EEC, with the exceptions of the GLP deviations listed in Appendix S. Deviations to the Protocol are also listed in Appendix S. In the opinion of the Study Director, no deviations were noted that would affect the conclusions of the study.

Study Director William J Breslin 10/9/98

William J. Breslin, Ph.D.

SIGNATURES

This report is being submitted by the following personnel:

William J. Breslin, Ph.D.
Director, Developmental and Reproductive Toxicology
Study Director

10/9/98
Date

C. Fred Morris, D.V.M., M.S., A.C.V.P. Diplomate
Vice President of Operations and
Director, Pathology Division

10/9/98
Date

KEY PERSONNEL

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Biostatistician Scientist

Information Technology
Gary N. Griffiths, Ph.D.
Director, Biostatistics and Information Technology

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1. QUALITY ASSURANCE STATEMENT

Below are the inspections conducted by the Quality Assurance Department and the dates the inspections were reported to the Study Director and Management:

Date(s) of Inspection	Study Phase Inspected	Date(s) Reported to Study Director/Management
10/30/97, 11/10/97	Protocol Review	9/16/98
11/4/97	Test Material Administration	12/5/97
11/11/97	Test Material Preparation	12/5/97
11/11/97	Prepared Test Material Sample Collection	12/5/97
11/11/97	Analytical Chemistry Operations	12/5/97
11/11/97	Litter Observations	12/5/97
11/19/97	Light Intensity Measurement	12/5/97
11/19/97	Litter Observations	12/5/97
12/8/97	Selection of F1 Males	1/9/98
12/22/97	Detailed Clinical Examination	1/9/98
1/14/98	Body Weight Measurement	3/6/98
1/14/98	Food Consumption Measurement	3/6/98
2/5/98	Necropsy	3/6/98
4/23/98	Sperm Analysis	6/2/98
4/28/98 to 5/5/98	Report Review	5/6/98, 6/2/98
5/15/98 to 5/20/98	Report Review	5/20/98, 6/2/98
5/21/98	Report Review	5/21/98, 6/2/98
4/28/98 to 5/21/98	Data Review	6/2/98
5/31/98 to 6/1/98	Report Review	6/2/98
9/9/98 to 9/10/98	Report Review	9/16/98
9/9/98 to 9/16/98	Report Review	9/16/98
10/6/98 to 10/9/98	Report Review	10/9/98

Anna J. Royal
Auditor, Quality Assurance

10/9/98
Date

2. SUMMARY

The objective of this study was to evaluate the effects of the test article (Bisphenol A, BPA) on male sexual development, as measured by sex organ weights, daily sperm production (DSP), epididymal sperm count and testis histopathology in the offspring of female mice exposed to the test article by deposition in the mouth on Days 11 to 17 of gestation. This study was conducted to investigate and further elucidate potential effects on male reproductive organs of low dose exposure to BPA during prenatal development applying a protocol for treatment as described by Nagel *et al.*, (1997) and vom Saal *et al.*, (1998). The study was not intended to establish either the maximum tolerated doses of BPA or define the upper bound of a no observed effect level as required in regulatory guidelines.

Groups of 28 females were administered 0.2, 2.0, 20 or 200 µg/kg/day of BPA in tocopherol-stripped corn oil, once daily on Days 11 to 17 of gestation. Two negative control groups of 28 mice/group were dosed with tocopherol-stripped corn oil only. An additional group of 28 females administered 0.2 µg/kg/day of diethylstilbestrol (DES) in the corn oil vehicle for the same time period served as a positive control. The dose level of DES was selected based on an expected maximum effect on the developing prostate as communicated by vom Saal (1996) and published by vom Saal *et al.*, (1997a). The 2.0 and 20 µg/kg/day doses of BPA were selected to repeat the exposure scenario used by vom Saal (1998) and Nagel *et al.*, (1997). The 0.2 and 200 µg/kg/day BPA dose levels were selected to provide additional information at a dose below and a dose above those chosen by vom Saal (1998) and Nagel *et al.*, (1997). Following the exposure period, the dams were allowed to deliver and raise their pups. On Day 22 of lactation, the litters were weaned, and a maximum of 4 males/litter were retained until 90 days of age. Following weaning, the adult females, all female pups, and extra male pups were euthanized. At 90 days of age, the male offspring (maximum of 4/litter) were euthanized and necropsied for evaluation of selected reproductive parameters. Following statistical comparison of the two negative control groups, these groups were combined into 1 negative control group for statistical evaluation of treatment related effects.

No treatment-related effects on clinical observations, body weight or food consumption were observed in adult females administered BPA at dose levels of 0.2, 2.0, 20, or 200 µg/kg/day. Similarly, no treatment-related effects on growth or survival of offspring from dams treated with 0.2, 2.0, 20, or 200 µg BPA/kg/day were observed. The total number of pups born per litter was statistically lower in the 200 µg BPA/kg/day group when compared to the combined controls. This difference was not attributed to treatment since the control value for this endpoint was higher than typically observed for this strain of mouse (approximately 9 pups/litter; Appendix Q), and the 200 µg/kg/day value for this endpoint was slightly higher than typically observed for this strain of mouse. In addition, the number of live pups born per litter was not statistically different when compared to the combined controls and this endpoint has not been reported to be affected at higher doses of BPA (Morrissey *et al.*, 1997). The male offspring of dams given 200 µg/kg/day BPA had mean values for cauda epididymis sperm concentration and daily sperm production which were less than, but not statistically different from, the values for the combined controls. There were no treatment related effects of BPA on prostate, preputial gland, seminal vesicle or epididymis weights at doses

previously reported to affect some of these organs (vom Saal *et al.*, 1998) or at doses an order of magnitude higher and lower. There were no effects on any parameter measured in females administered DES at a dose level of 0.2 µg/kg/day. There were also no effects on the growth or sexual development of male offspring of dams exposed to DES during gestation.

In conclusion, the effects reported by vom Saal *et al.*, (1997a) on the prostate weight of male offspring from dams exposed to DES during gestation were not observed in the present study. The absence of effects on the prostate weight, other reproductive organ weights, sperm count or daily sperm production of male offspring from dams exposed to BPA at doses equivalent to those used previously and at an order of magnitude higher and lower were in contrast to those findings previously reported by Nagel *et al.*, (1997) and vom Saal *et al.*, (1998).

3. INTRODUCTION

3.1. Objective

Previous experiments by Nagel *et al.*, (1997) and vom Saal *et al.*, (1998) reported that exposure of pregnant females to BPA via deposition into the mouth on Days 11 to 17 of gestation produced alterations in prostate weight, other reproductive organ weights, or sperm parameters of male offspring. The objective of this study was to investigate and further elucidate the potential effects of the test article (Bisphenol A, BPA) on sexual development, as measured by sex organ weights, daily sperm production (DSP), epididymal sperm count and testis histopathology in the male offspring of female mice exposed to the test article by deposition in the mouth on Days 11 to 17 of gestation.

3.2. Species Selection

Mice of the CF-1 strain were employed because this species and strain was used in the work of Nagel *et al.*, (1997) and vom Saal *et al.*, (1998). In addition, the mouse is a universally used model for evaluating toxicity of various classes of chemicals and for which there is a large historical database.

3.3. Study Schedule

Study Initiation (Protocol Signed by the Study Director)	September 24, 1997
Protocol Approved by the Sponsor	October 14, 1997
Arrival of Animals	October 20-24, 1997 October 27-31, 1997 November 3-7, 1997 November 10-11, 1997
Randomization	October 30-31, 1997 November 1-3, 1997 November 6-10, 1997 November 13-15, 1997
Commencement of Treatment	October 31, 1997
End of Treatment	November 22, 1997
Necropsy of Male Offspring	February 5 through 24, 1998
In-life Completion Date	February 24, 1998
Final Draft Report	September 3, 1998

4. MATERIALS AND METHODS

4.1. Experimental Design

4.1.1. Animal Receipt and Maintenance

On October 20-24, October 27-31, November 3-7, and November 10-11, 1997 a total of 444 female CF-1 mice were received from Charles River Laboratories, Portage, MI (384 mice were ordered; 444 mice were shipped and received). Animals were received time mated from the supplier on gestation Day 0. The day of mating was considered gestation Day 0. Upon receipt at the laboratory, the mice were housed individually. During the 11-day acclimation period, all mice were weighed on Day 0 and 10 of gestation, observed daily for any clinical signs of disease, and given a detailed physical examination prior to the start of the study. Mice considered suitable for study were randomly selected and divided into groups as described in section 4.1.2. of the report.

In addition, on November 24, 1997 a total of 10 male CF-1 mice were received from Charles River Laboratories, Portage, MI. The prostate of 8 of these males was evaluated microscopically following prosection to determine the impact of the prosection technique of Nagel *et al.*, (1997) on the microscopic integrity of this tissue.

Throughout the study, all mice were kept in an environmentally controlled room. Temperature and relative humidity were monitored and recorded daily and maintained between 69°F (21°C) and 75°F (24°C) and 43% and 65%, respectively. Fluorescent lighting provided illumination 12 hours per day via an automatic timer. Lighting levels were maintained below 18 ft-candles, as measured from 1 meter off the floor, approximately 1-6 inches in front of the cages on each side of the rack. Light intensity was monitored and recorded 6 times throughout the study. Low volume music was played in the animal rooms to provide background noise to reproduce the environmental conditions used by vom Saal (1996). Diet (Certified Rodent Chow #5002, PMI Feeds, Inc., St. Louis, Missouri) and drinking water was available *ad libitum*. Water was available via glass bottles during the exposure period. Teflon seals were used with the lids of the water bottles. Females were housed individually throughout the study except during lactation when they were housed with their litters. Adult females and weanling males were individually housed in polypropylene plastic tubs with stainless steel lids and corn cob bedding. Males selected to be retained to 90 days were individually housed following weaning in suspended, stainless steel, wire-mesh type cages. Feed containers and water bottles were changed and sanitized once per week. Tubs and bedding were also changed weekly.

Certification analysis of each lot of diet was performed by the manufacturer. The same lots of diet were provided to animals from all groups at the same time during the course of the study to control across groups for possible variation in the content of the diet. The drinking water used for the test animals was monitored for specified contaminants at periodic intervals according to Standard Operating Procedures of MPI Research. The Study Director was not

aware of any potential contaminants present in the water that would interfere with the results of this study.

4.1.2. Assignment to Study

Following arrival of time mated females to the laboratory, pretest body weights were obtained on days 0 and 10. Animals gaining ≥4.5 grams in body weight during the gestational day 0 to 10 pre-exposure interval were randomized into seven groups on Day 10 of gestation using a stratified, by weight, block randomization procedure until 28 mice/treatment group were assigned to test. The weight gain criterion was intended to eliminate placement of non pregnant females on the study. The treatment of mice was stagger-started over a period of 17 days in 13 subgroups for each treatment group to facilitate animal treatment and handling during gestation, delivery, and necropsy.

Animals were assigned to study as shown below.

Assignment to Study			
		Number of Animals	
Group	Dose Level µg/kg/day	Selected Females [a]	Selected F_1 Males [b]
1	Negative Control	28	65
2	Negative Control	28	94
3	0.2 DES	28	80
4	0.2 BPA	28	84
5	2.0 BPA	28	75
6	20 BPA	28	70
7	200 BPA	28	80

[a] 28 females/group were dosed on gestation Days 11-17.

[b] At weaning, the surviving males (maximum of 4 per litter) from each litter were continued on study, untreated until 90 days of age.

Each animal was identified by cage, group, and individually by a metal ear tag bearing the animal number. The individual animal number plus the study number of the testing facility comprised a unique identification number for each animal. Weanlings were identified by tail tattoo. Prior to weaning, pups were not individually identified.

4.2. Test, Negative and Positive Control Articles

4.2.1. Test Article Identification

The test article, 4,4'-isopropylidene-2-diphenol (Bisphenol A, BPA, CAS# 80-05-7) was received from the Sponsor. The Sponsor provided BPA with the purity of ≥ 99%. Analysis of BPA for purity was conducted by The Dow Chemical Company, Midland, MI. The purity analysis included identification of impurities present at a concentration of 0.1% or greater. Pertinent information is presented in Appendix A.

4.2.2. Negative Control Article Identification

The negative control was tocopherol-stripped corn oil (>99% purity), received from ICN Biomedicals Inc., Aurora, Ohio.

4.2.3. Positive Control Article Identification

The positive control, diethylstilbestrol (DES) with a purity of 99%, was received from Sigma Chemical Co., St. Louis, MO. Pertinent information is presented in Appendix A.

4.2.4. Test Article and Negative (Vehicle) Control Preparation

No adjustments were made for purity of the BPA as received from the Sponsor. Appropriate amounts of BPA were mixed with tocopherol-stripped corn oil to achieve the desired concentrations. Fresh solutions were prepared for each concentration weekly. The dose solutions were prepared and stored in glass containers. The concentration of the solutions were prepared such that a dose volume of approximately 30µL/mouse would deliver the appropriate dose on a µg/kg basis. Based on the expected body weight of 40 grams for pregnant CF-1 mice at the midpoint of dosing (gestation Day 14), the dose volume on a mL/kg body weight was equal to 0.75 mL/kg. Aliquots of all dosing solutions were stored until completion of the study, after which the retained aliquots were discarded.

4.2.4.1. Negative (Vehicle) Control Article Preparation

The vehicle (70 ml of tocopherol stripped corn oil) was dispensed into amber glass containers prior to handling the test article and stored at room temperature.

4.2.4.2. Test Article Stock Solution Preparation

BPA was ground using a mortar and pestle. The required amount of ground BPA was weighed directly into a beaker. Vehicle was added to the beaker and the contents were mixed using a magnetic stir bar and stir plate. The contents of the beaker were stirred while being heated in a water bath at 62°C until dissolved (on October 8, 1997, sonication of the stock solution being prepared for Day 0 stability analysis for dose Groups 4, 5, 6, and 7 was necessary to aid in dissolution of the contents of the beaker). The solution was transferred into a graduated cylinder and additional vehicle was added to yield 90 ml of prepared stock solution. The cylinder was shaken manually and the contents were dispensed into an amber glass container. The contents of the container were stirred using a magnetic stir bar and stir plate and stored at room temperature.

4.2.4.3. Test Article Dose Solution Preparation

While the BPA stock solution was stirring, using a magnetic stir bar and stir plate, the required amount of BPA stock solution was withdrawn using a glass syringe (40 µl for Group 4, 400 µl for Group 5, 4 mL for Group 6, and 40 mL for Group 7) and transferred into a

graduated cylinder. Vehicle was added to the cylinder to yield the required volume of prepared dose solution(s). The cylinder was shaken manually and the contents were dispensed into a beaker. The contents of the beaker were stirred using a magnetic stir bar and stir plate and were dispensed, using a glass syringe (10 mL per container for all groups), into amber glass containers and stored at room temperature.

4.2.5. Positive Control Stock and Dose Solution Preparation

The positive control test article (DES) was used as received and no adjustments were made for purity. Appropriate amounts of DES were mixed with tocopherol-stripped corn oil to achieve the desired concentrations. Fresh solutions were prepared weekly. The dose solutions were prepared and stored in glass containers. The concentrations of the dose solutions were prepared such that a dose volume of approximately 30µL/mouse would deliver the appropriate dose on a µg/kg basis. Based on the expected body weight of 40 grams for pregnant CF-1 mice at the midpoint of dosing (gestation Day 14), the dose volume used on a mL/kg body weight was equal to 0.75 mL/kg. Aliquots of all dosing solutions were stored until completion of the study, after which the retained aliquots were discarded.

4.2.5.1. Positive Control Stock Solution Preparation

The required amount of DES was weighed directly into a beaker. Appropriate amounts of vehicle were added and the contents were mixed using a magnetic stir bar and stir plate. The contents of the beaker were stirred while being heated in a water bath at 62°C until dissolved. The solution was transferred into a graduated cylinder and additional vehicle was added to yield 100 mL of prepared stock solution. The cylinder was shaken manually and the contents were dispensed into an amber glass container. The contents of the container were stirred using a magnetic stir bar and stir plate and stored at room temperature.

4.2.5.2. Positive Control Dose Solution Preparation

The DES dose solutions were prepared weekly, prior to dosing, and stored in glass containers. 240 µL of DES stock solution was transferred into a graduated cylinder using a (500µL) syringe while stirring the stock solution using a magnetic stir bar and stir plate. Vehicle was added to the cylinder to yield 90 mL of prepared positive control dose solution. The cylinder was shaken manually and dispensed into a beaker. The contents of the beaker were stirred using a magnetic stir bar and stir plate and dispensed, using a glass syringe (10 mL per container for all groups), into amber glass containers. The glass containers were capped using a black cap with a teflon liner and stored at room temperature.

4.2.6. Test Article, Vehicle Control and Positive Control Dose Solution Analysis

Only the analytical results from the dose solutions that were within the specifications required by the protocol and therefore administered to the animals are presented in the report. BPA dosing solutions were prepared once weekly according to the test material preparation procedures presented in Section 4.2.4.3., with the following exceptions:

1) The initial Week 1, Group 4 dose solution was prepared on 10/28/97, analyzed and determined to be less than 90% of the targeted concentration (outside of the SOP and Protocol specifications). This solution was discarded and a new group 4 dose solution was prepared and analyzed on 10/30/97 according to the procedures presented in Section 4.2.4.3.

2) The initial Week 2, Group 4 dose solution was prepared on 11/4/97, analyzed and determined to be less than 90% of the targeted concentration. This solution was discarded and a new group 4 dose solution was prepared and analyzed on 11/5/97 according to the procedures presented in Section 4.2.4.3.

3) The initial Week 4, Group 4 dose solution was prepared on 11/19/97, analyzed and determined to be less than 90% of the targeted concentration. This solution was discarded and a new group 4 dose solution was prepared on 11/20/97 according to the procedures presented in Section 4.2.4.3. The second preparation of this solution was also analyzed and determined to be less than 90% of the targeted concentration. The second dose solution preparation was retained. A third mix of the Group 4 dose solution was prepared on 11/20/97 using the procedures presented in Section 4.2.4.3. This third mix was analyzed and determined to be less than 90% of the targeted concentration and, therefore, was discarded. The retained second dose solution preparation was "spiked" on 11/20/97 with a quantity of the BPA stock solution required to bring the solution to within the targeted concentration. However, subsequent analysis determined that this retained, "spiked", second preparation was greater than 110% of target (out of SOP specifications). The retained, "spiked", second preparation was then diluted on 11/21/97 with the appropriate amount of corn oil vehicle to bring the solution down to the targeted concentration. This solution was again analyzed and found to be within specifications. The Week 4, Group 4 dosing solution was administered for only one day to the last of the 13 subgroups of animals placed on study.

DES dosing solutions were prepared once weekly according to the test material preparation procedures presented in Section 4.2.5.2., with the following exceptions. The initial Week 1 (preparation date 10/28/97) and Week 2 (preparation date 11/4/97) DES dosing solutions were analyzed and determined to be less than 90% of the targeted concentration. Both of these solutions were then "spiked" ("spike" dates 10/30/97 and 11/6/97, for Weeks 1 and 2, respectively) with a quantity of the DES stock solution required to bring the dosing solutions to within their targeted concentration. The "spiked" solutions were reanalyzed and determined to be within ±10% of the targeted concentrations.

4.2.7. Homogeneity

Prior to initiation of dosing, trial batches of the 0.2 (low), and 200 (high) µg/kg/day BPA dose solutions were prepared (9/26/97) according to the methods described in Section 4.2.4.3. Duplicate samples were collected from the top, middle and bottom of each solution and analyzed using Method 1 as described in Appendix B.

Prior to initiation of dosing, trial batches of the DES dose solution were prepared on 9/26/97, 10/3/97 and 10/8/97 according to the procedures described in Section 4.2.5.2., analyzed and determined to be outside the SOP specification for concentration (< 90% of targeted concentration). The DES dose solution was prepared a fourth time on 10/13/97 using the same preparation procedures, analyzed and determined to be within specification. For each analysis, duplicate samples were collected from the top, middle and bottom of each solution and analyzed using Method 1 (solutions mixed on 9/26/97, 10/3/97 and 10/8/97) or Method 2 (solution mixed on 10/13/97) as described in Appendix B.

4.2.8. Stability

Initial trial batches of each BPA dosing solution were prepared on 9/26/97 for stability analyses according to the method described in Section 4.2.4.3. The Group 4 and Group 7 preparations were also used for homogeneity analysis. Following analysis, the Group 4 and Group 7 dosing solutions were determined to be within ±10% of the targeted concentrations; the Group 5 and Group 6 solutions were determined not to meet the SOP specification of ± 10% of the targeted concentration. The new Group 5 and 6 dose solutions were prepared on 10/3/97 and analyzed along with the Group 4 and 7 dose solutions (day 7). At this second analysis, the Group 5 and 6 dose solutions were out of specifications for concentration and the Group 4 and 7 dose solutions were out of specification for 7-day stability (day 7 concentration ±10% of day 0 concentration). All BPA dose solutions were prepared again on 10/8/97 using the method described in Section 4.2.4.3. and reanalyzed on 10/9/97. All of these dose solutions were determined to be within concentration and stability specifications, with the exception of the Group 4 solution which did not meet the 10-day stability criteria. The Group 4 10-day stability concentration was less than 90% of the day 0 concentration, but was within 10% of the targeted concentration.

Prior to initiation of dosing a trial batch of the DES dosing solution was prepared on 10/13/97 for stability analyses according to the method described in Section 4.2.5.2. The dose solution was analyzed on the day of preparation (Day 0) and at 4, 7 and 10 days after preparation using Method 2 as described in Appendix B.

4.2.9. Test Article, Vehicle Control, and Positive Control Administration

The control article, positive control article (in vehicle) and test article (in vehicle) were administered by deposition into the mouth using a micropipetter as reported by Nagel *et al.* (1997). Only dose solutions assaying at nominal ±10% of the targeted concentrations were used for dosing.

All doses were adjusted daily, based on body weight, to provide constant dose levels of 0, 0.2, 2.0, 20 and 200 µg/kg/day BPA or 0.2 µg/kg/day DES. The dose volume administered was 0.75 mL/kg body weight. This volume was equivalent to approximately 30 µL/mouse/day, based on an average weight of a 40 g/mouse. The control animals received the vehicle at a dosage volume comparable to that received by the test animals. Individual dosages were based on the most recent body weights.

4.2.10. Justification of Dosage Levels

The 0.2 µg/kg/day DES positive control and the 2.0 and 20 µg/kg/day doses of BPA were selected to repeat the exposure scenario used by vom Saal (1996) and Nagel *et al.*, (1997). The 0.2 and 200 µg/kg/day BPA dose levels were selected to provide additional information at a dose below and a dose above those chosen by vom Saal (1998) and Nagel *et al.*, (1997).

4.3. Observations

4.3.1. Cageside Observations

All mice were observed at least twice a day, 7 days a week, for morbidity, mortality, and signs of injury. All findings were reported. Findings of mortality or other signs of toxicity were recorded on the day they were observed.

4.3.2. Detailed Clinical Examinations

During treatment (Days 11-17 of gestation), a detailed clinical examination of each mouse was performed once daily at the time of dosing, except on November 10, 1997, when the examinations were conducted approximately 6 hours after dosing. After treatment was terminated, detailed clinical examinations were conducted on a weekly basis. Following weaning and transfer into individual housing, each weaned animal was given a detailed clinical examination on the day of weaning, daily for the 4 consecutive days following weaning, and weekly thereafter until study termination. Examinations included observations of the general condition, activity, and behavior; examination of excretory matter; respiration; surface signs (scabbing, hair loss); and oral, nasal and ocular regions. Modifiers were included in the clinical sign when necessary to describe the location, size, shape, color or other characteristics.

4.3.3. Food Consumption

Food consumption in time-mated females was recorded during the gestation interval of Days 0 to 7, 7 to 10, and 10 to 11. Following selection of females to be placed on study, food consumption was measured during the gestation interval of Days 11 to 17. After parturition, food consumption was recorded twice during the first and second weeks of lactation and at 2- to 3-day intervals during the last week of lactation. For postweaning males that were retained

until 90 days of age, food consumption was recorded weekly at the time each body weight was recorded. Food containers were weighed at the start (full weight) and end (remaining weight) of a measurement interval.

4.3.4. Body Weights

Time-mated females were weighed on Days 0, 10 and 11 through 18 of gestation. Females that delivered litters were weighed on Days 1, 4, 7, 14 and 21 of lactation. Male and female pups were weighed individually on Days 1, 4 and 22 of lactation. Each male pup selected to be maintained to 90 days of age were weighed on the day of weaning (Day 22 of lactation) and weekly thereafter.

4.3.5. Litter Data, Culling, and Weaning

All litters were examined as soon as possible after delivery. The day of parturition (Day 0) was recorded for each litter. The number of live and dead pups and sex and weight of each pup were recorded for each litter on Days 1, 4 and 22 postpartum. In addition, any visible physical abnormalities or demeanor changes during lactation were recorded for pups in each litter. To reduce the variation in the growth of pups, the litters with a total number of pups exceeding 8 were culled on Day 4 postpartum. Culled litters were reduced to a total of 8 pups (8 males when possible). If fewer than 8 males were available, the appropriate number of females were retained to achieve a total of 8 pups. Pups to be culled were selected using a computer-generated randomization procedure. Litters with 8 or fewer pups were not culled. Preferential culling of runts was not performed. Culled pups were examined externally for abnormalities and euthanized by the deposition of a pentobarbital solution into the oral cavity.

Weaning of all litters were performed 22 days after delivery. A maximum of 4 male weanlings per litter were randomly selected, using a computer-generated procedure, to continue on test to 90 days of age. Weanlings not held for maintenance to 90 days of age were examined externally for abnormalities and euthanized by carbon dioxide inhalation and discarded.

4.4. Anatomic Pathology

A complete necropsy was performed by trained personnel under the direct supervision of a veterinary pathologist on all male offspring selected to be retained to 90 days of age. The scheduled necropsy was performed at 90±2 days of age. The males scheduled for necropsy at 90 days of age were removed from their cage, verified for proper identification, anesthetized with carbon dioxide and euthanized by decapitation. The method of anesthesia, euthanasia, and dissection of reproductive organs used was consistent with the methods of vom Saal and Thayer (1997). Coagulating glands, epididymis (left), prostate, and seminal vesicles were collected and saved from these mice and preserved in neutral, phosphate-buffered 10% formalin. The left testis was preserved in 1.5% gluteraldehyde/4 % formaldehyde fixative.

Adults and retained F_1 male weanlings which died on study or were sacrificed moribund were necropsied in an attempt to determine the cause of death; however, terminal body weights, organ weights, and tissues were not obtained. Moribund animals were euthanized by carbon dioxide inhalation. Pups found dead or euthanized *in extremis* prior to weaning were examined to the extent possible and discarded. These pups were euthanized by deposition of a pentobarbital solution into the oral cavity. All adult females sacrificed for necropsy were euthanized by carbon dioxide inhalation.

Prior to the time of necropsy of the 90-day-old animals, 8 additional untreated male mice, approximately 60 days of age, purchased from Charles River Laboratories, Portage, MI, were anesthetized by carbon dioxide inhalation. The mice were then euthanized by decapitation and exsanguinated. The prostate was removed using the technique described below (4.5. Dissection of Reproductive Organs) and preserved in neutral, phosphate-buffered 10% formalin for the purpose of determining if the prosection technique caused mechanical damage or provided histologic artifacts. The prostate was then processed, embedded in paraffin, stained with hematoxylin and eosin, and evaluated histologically.

4.5. Dissection of Reproductive Organs

Dissection of the male reproductive organs was carried out by trained personnel using the technique described below (vom Saal and Thayer, 1997b). These personnel were blind to treatment of each group during the dissection.

The preputial glands were carefully dissected free of fat and connective tissue to the point where they enter the urethra. The preputial glands were then pressed together under a 5,000-g weight for approximately 30 seconds (±5 seconds) to remove excess fluid and then weighed together. The testes with attached epididymides were removed. The epididymides were trimmed free from the testes and each testis (left and right) were weighed separately. The right testis was frozen at approximately -70°C for daily sperm production to be performed at a later time. The left testis was fixed in 1.5% gluteraldehyde/4% formaldehyde. The right caudal epididymis was removed, weighed, and was frozen at approximately -70°C for epididymal sperm count. The left epididymis and the remainder of the right epididymis was fixed in 10% neutral buffered formalin.

The abdominal cavity was then opened to expose the abdominal sex accessory organs. The coagulating glands were carefully separated from the seminal vesicle by blunt dissection and collected. The seminal vesicles were carefully separated from the ventral prostate by teasing the prostate back from the seminal vesicle to the point where the seminal vesicle entered the urethra, being careful not to rupture them. The seminal vesicle were then pressed together under a 5000-g weight for approximately 30 seconds (±5 seconds) to remove the fluid and then weighed. Both the coagulating glands and seminal vesicles were fixed in 10% neutral buffered formalin. If the coagulating glands or seminal vesicle were ruptured at necropsy, the prosector cleaned as much fluid as possible off the prostate. The rupture of these organs was documented in the pathology record. The ventral and dorsal lobes of the prostate were carefully teased free of fat and connective tissue. The entire prostate was removed, including

the portion of the urethra between the ventral and dorsal lobes of the prostate. The urethra was gently removed from the area between the prostate lobes. The prostate was then checked to ensure that all the fat was removed, weighed, and placed in 10% neutral buffered formalin.

Of the males selected to be retained to 90 days of age, 10 mice per dose group were randomly selected as follows to preserve the prostate for possible histologic evaluation by "conventional" prostate prosection (i.e., removal of urinary bladder and abdominal sex accessory organs by transection of pelvic urethra and fixation without further dissection). This was carried out because the histologic evaluation of prostates from stock animals dissected using the procedure described above resulted in mechanical damage and histologic artifacts. For dose groups with >10 litters with 4 surviving pups per litter, 10 litters with 4 surviving pups per litter were randomly selected. From these 10 randomly selected litters, 1 pup per litter was randomly selected. For dose groups with ≤10 litters with 4 surviving pups per litter, 1 pup from each litter with 4 surviving pups was randomly selected. If less than 10 litters with 4 pups were available, additional litters were randomly selected from litters with 3 surviving pups per litter until a total of 10 litters per group were obtained. From these 10 litters per group, 1 pup per litter was randomly selected. The prostate from these 10 males per dose group were removed according to MPI Standard Operating Procedures and preserved in neutral, phosphate-buffered 10% formalin. Histological evaluation of these prostates were not carried out since no treatment related change in prostate weights were observed.

4.6. Organ Weights

The following organs of all 90-day old male offspring were weighed (see list below). The seminal vesicles and preputial glands were pressed/blotted by putting the organs between paper towels and placing a 5,000 g weight on the organs for 30 seconds (±5 seconds). Both absolute and relative (to body weight and to brain weight) weights were reported. The prosector(s) removed the organs blind to treatment.

- right and left testes (separate)
- epididymides (together)
- right cauda epididymis
- seminal vesicles without coagulating glands and their fluids (the seminal vesicles were pressed/blotted to void their fluids)
- prostate (the prostates from the 10 males per dose group selected to undergo the "conventional" prostate prosection were not weighed.)
- brain
- liver
- kidneys (together)
- preputial gland (without fluid, pressed/blotted)

4.7. Daily Sperm Production

Daily sperm production was determined following the general procedures outlined by Blazak *et al.*, (1993), as revised by Buchanan (Waechter, 1997). In summary, the right and left testis were weighed separately at the time of necropsy. If the testes were not equivalent with respect to size, weight, or macroscopic appearance, this was noted and the animal removed from the study for daily sperm production determination. The right testis was then decapsulated, placed in a measured volume of homogenization media (Saline [8.77 g NaCl /Liter] and .05% Triton X-100) and homogenized. An aliquot of the homogenate was evaluated with a hemocytometer for the number of homogenization-resistant sperm heads.

Step 17-19 spermatids survive the homogenization procedure and can be counted in the hemocytometer. In the mouse, developing spermatids typically spend 4.84 days in steps 17-19 (Clermont and Harvey, 1965). Thus, DSP is calculated by dividing the number of spermatids per testis by 4.84. The formula used for determination of daily sperm production is listed below.

Daily sperm production = (average hemocytometer count x dilution factor x hemocytometer volume correction factor) / spermatogenesis time divisor.

Spermatogenesis time divisor = the amount of time (days) during spermatogenesis that the spermatids are resistant to homogenization.

The dilution factor = A x B
A = total volume of homogenate
B = (volume of stain + volume of homogenate aliquot) / volume of homogenate aliquot

4.8. Epididymal Sperm Count

If the testes were not equivalent with respect to size, weight, or macroscopic appearance, this was noted and the animal removed from the study for epididymal sperm count determination. The right cauda epididymis was weighed, placed in a measured volume of physiologically buffered saline (PBS), and homogenized. An aliquot of the sperm/saline mixture was then counted in a hemocytometer. The hemocytometer count was multiplied by appropriate volume and dilution factors to give a total cauda epididymal sperm count. A sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. The formula used for determination of epididymal sperm count per gram of tissue is listed below.

Sperm count per gram of tissue = (average hemocytometer count x dilution factor x hemocytometer volume correction factor) / grams of epididymal tissue.

The dilution factor = A x B
A = total volume of homogenate
B = (volume of stain + volume of homogenate aliquot) / volume of homogenate aliquot

4.9. Histopathology

Histologic examination of the left testis of each 90-day old male from all exposure groups was performed. The left testis was embedded in glycol methacrylate, processed to slides stained with periodic acid Schiff and hematoxylin, and microscopically evaluated. The right testis was not evaluated histologically because it was used to determine daily sperm production.

The prostate from the 8 additional untreated male mice were processed, embedded in paraffin, stained with hematoxylin and eosin, and evaluated histologically. The objective of evaluating the prostate of the additional, untreated male mice was to determine if the prostate prosection technique used in this study caused mechanical damage or induced histologic artifacts, such that effective histopathologic evaluation of this tissue was compromised.

4.10. Statistical Analysis

The table below defines the set(s) of comparisons used in the statistical analyses.

Statistical Comparisons	
Group(s)	Group(s)
1	2
1 and 2 combined	3, 4, 5, 6, 7

Descriptive statistics (means and standard deviations) were reported for food consumption. Body weights, gestation/lactation body weight gains, organ weights, sperm counts, sperm production, and litter size were analyzed using the litter as the experimental unit (Haseman and Kupper, 1979). These data were first analyzed by the Levene's test (Milliken and Johnson, 1992). If the Levene's test was not significant ($p \geq 0.01$), an analysis of variance (ANOVA) was run. If the ANOVA was significant ($p \leq 0.05$), a Dunnett's test (Dunnett, 1955) was performed. If the Levene's test was significant ($p < 0.01$), a rank transformation was performed on these data and an ANOVA was run. If the ANOVA was significant ($p \leq 0.05$), the Wilcoxon Rank-Sum Test (Connover, 1980) with Bonferroni's correction was performed.

Fertility indices were analyzed by the Fisher exact probability test (Agresti, 1990) and Bonferroni's correction was used for multiple testing of groups in comparison to a single control. Evaluation of the neonatal sex ratio was performed by the binomial distribution test (Gill, 1978). Survival indices and other incidence data among neonates were analyzed using the litter as the experimental unit by a non-parametric ANOVA. If the ANOVA was significant, the Wilcoxon Rank-Sum Test with Bonferroni's correction was performed. The nominal alpha level used was 0.05.

Because numerous measurements were statistically compared in the same group of animals, the overall false positive rate (Type I errors) was much greater than the cited alpha levels would suggest. Thus, the final interpretation of numerical data considered statistical analyses

along with other factors, such as dose-response relationships and whether the results were significant in the light of other biologic and pathologic findings.

4.11. Data and Specimen Retention

All raw data, documentation, records, protocol, reserve samples, specimens (slides, blocks, and wet tissues), and the final report generated as a result of this study will be retained at MPI Research for a period of 5 years following completion of the study (final report issue date). Retention of materials after the times stated above will be subject to future contractual agreements between the Sponsor and MPI Research.

5. RESULTS

5.1. Test Article, Vehicle Control, and Positive Control Dose Solution Analysis

5.1.1. Homogeneity

Homogeneity results for test article and positive control article in dose solutions are presented in Appendix B.

Homogeneity analyses were conducted on the Group 3, 4 and 7 dose solutions. BPA and DES were found to be homogeneously distributed throughout the corn oil vehicle. The criteria for homogeneity acceptance was ±10% of the target (nominal) concentration with a relative standard deviation (RSD) of ≤ 10%.

5.1.2. Stability

Stability data for test article and positive control article in dose solutions are presented in Appendix B.

The stability of BPA and DES in dose solutions over a 7- and 10-day storage period was determined by HPLC analysis prior to the initiation of treatment. The results of the 10-day stability were: 97%, 84%, 107%, 100% and 107% for Groups 3 through 7, respectively. Thus, the Group 5, 6, and 7 BPA dose solutions and Group 3 DES dose solution were found to be ±10% of their Day 0 concentration and were considered to be stable in corn oil for up to 10 days. The Group 4 BPA dose solution was <90% of its Day 0 concentration and was not determined to be stable following 10 days of storage. However, the Group 4 BPA dose solution on Day 7 was 92% of its Day 0 concentration and was determined to be stable for at least 7 days.

5.1.3. Periodic Analysis for Concentration

Periodic analysis data for test article and positive control article in dose solutions are presented in Appendix B.

Concentration analyses for BPA and DES in dose solutions were conducted each week prior to the use of dose solutions for treatment. The average concentrations ranged from 93 to 109% of the nominal values. Thus, the concentrations of BPA and DES in dose solutions were found to be ±10% of the nominal concentration as specified in the protocol.

5.2. Observations

5.2.1. Cageside and Detailed Clinical Examinations

Clinical observations during gestation and lactation are summarized in Tables 1 and 2. Individual female appearance and observations are presented in Appendix C.